Electronic Supplementary Material (ESI) for Dalton Transactions.

Supporting Information for

Direct Detection of Iron Clusters in L Ferritins through ESI MS Experiments

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Primary sequences alignment

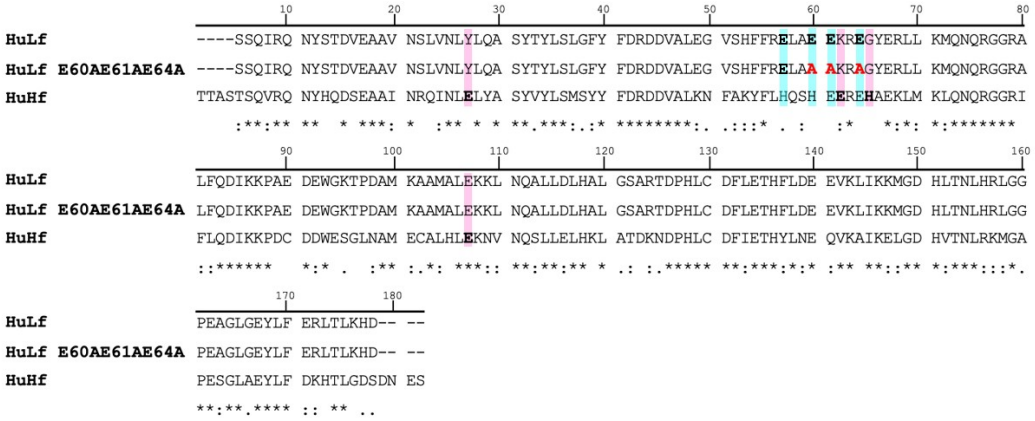

Figure S1. Multiple sequence alignment among HuLf, HuLf-E60AE61AE64A and HuHf aminoacidic sequences was performed by Clustal Ω. The cyan-colored boxes highlight the localization of the nucleation site while the pink-colored boxes are related to the ferroxidase site. The black bold letters help showing the residues belonging to each kind of sites, the red ones refer to the inserted mutations. The residue numbering of HuHf is used as standard sequence numbering for all ferritins.

Structural visualization of the nucleation and ferroxidase sites

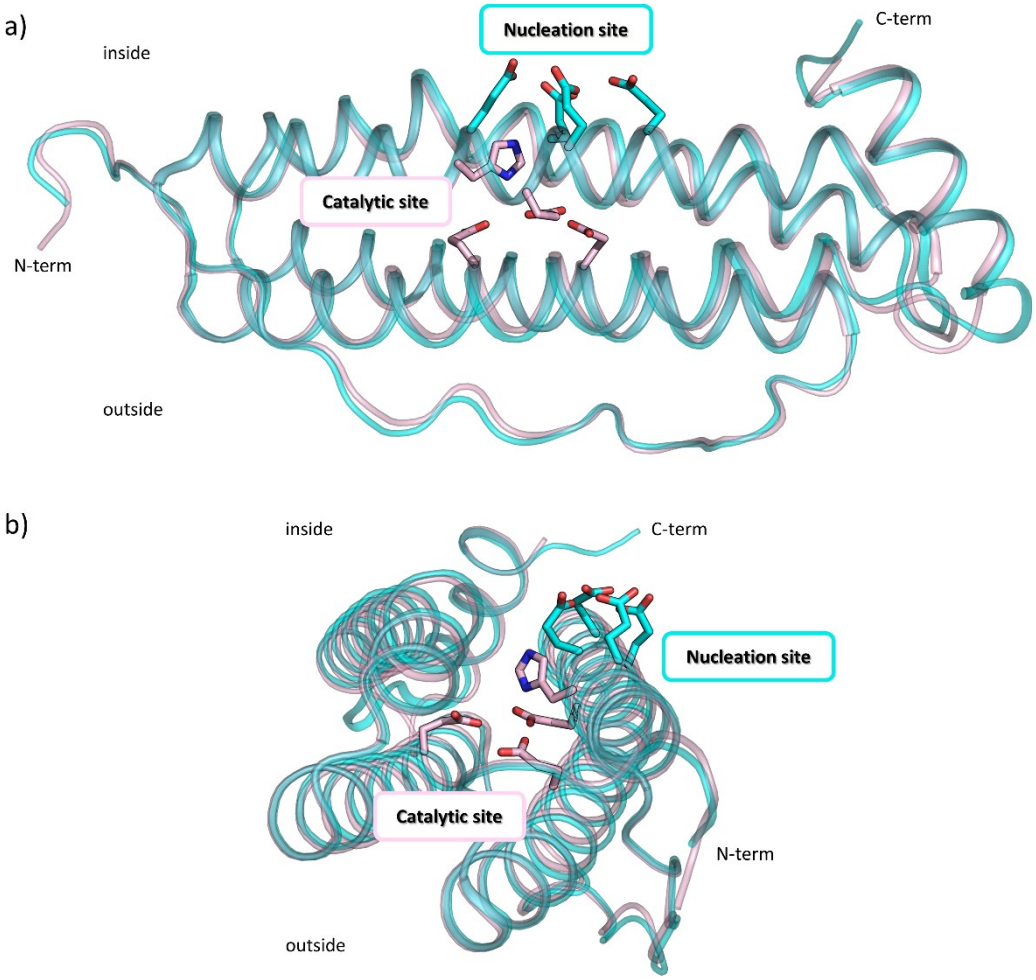

Figure S2. a) Superimposition of HuLf (PDB id: 5LG8)[1] and HuHf (PDB id: 4ZJK)[2] monomer structures represented as cyan and pink cartoon, respectively. In sticks are shown the iron binding residues at the nucleation site in HuLf, placed on the internal surface where the iron biomineral starts to grow, and at the catalytic site in HuHf, localized in the middle of the α-helices bundle, which is deputed to the oxidation of incoming iron ions. Carbon atoms colors are the same as the corresponding protein cartoon monomer and oxygen and nitrogen atoms are colored red and blue, respectively. b) Side view of the protein subunits that allows to better appreciate the positions of the two key sites evidenced in the structures.

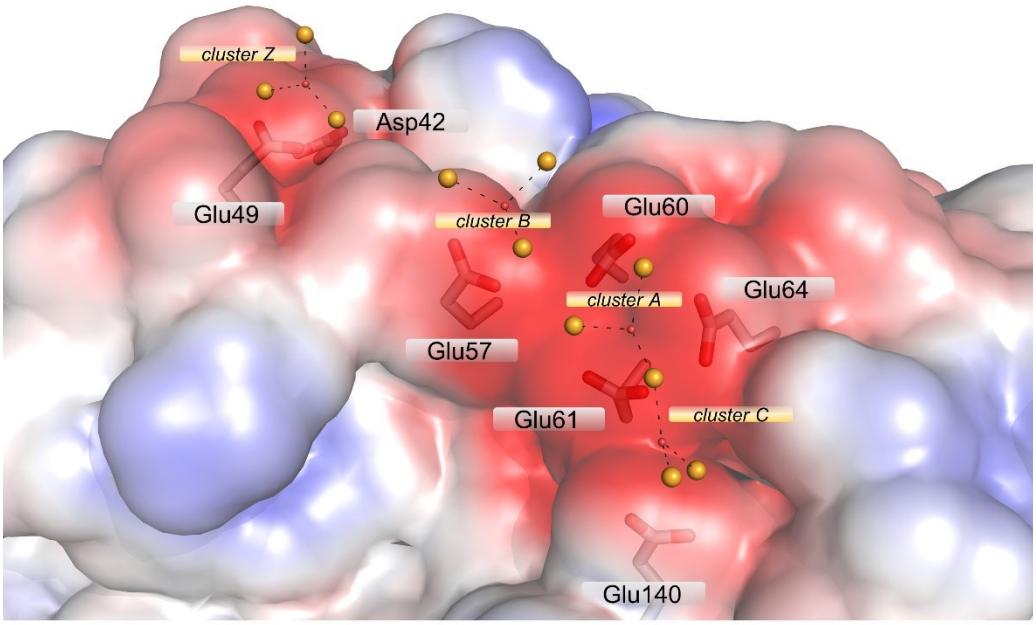

Figure S3. Electrostatic surface representation of the inner cavity of HuLf (calculated on the structure of HuLf after 30 min Fe^{3+} exposure, PDB id: 6TSA). In orange spheres the iron atoms, named depending on the cluster they belong to (cluster A, FeA1-3; cluster B, FeB1-3; cluster C, FeC1-2,FeA2 and cluster Z, FeZ1-3). In sticks, all the iron binding residues, including Asp42 and Glu49 at the cluster Z that transiently appears only at 30 min of iron diffusion.

Ferritin samples preparation

The expression and purification of homopolymeric wild type HuLf, HuLf-E60AE61AE64A variant and HuHf proteins were performed accordingly to reported protocols.[1–3] All the protein samples were deprived of iron or any other metals taken from the culture media, making the ferritins apo as published elsewhere.[4] For ESI-MS analysis each protein was prepared in the apo form, to register the reference spectrum, and in the holo form (loaded with iron ions). Mineralization of ferritin samples dissolved in 100 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 100 mM NaCl pH 7 buffer with variable amounts of iron (in the range 72-480 Fe(II) per cage) was obtained adding a proper amount of a fresh ferrous sulfate stock solution in 1 mM HCl. The samples were rapidly mixed and incubated 2 hours at room temperature, then stored over night at 4 °C. After that, a centrifugation step was performed to remove precipitates and the iron ions not captured into the cages were removed with extensive ultrafiltration by using centrifugal devices (cut off 50 kDa). Finally, the apo and the iron-loaded ferritins were buffer exchanged into 20 mM ammonium acetate at pH 6.8 and their concentrations determined by Bradford assay.

ESI-MS experiments

The protein solutions were further diluted with 2mM ammonium acetate solution, pH 6.8, to a final protein concentration of 10^{-6} M. 0.5% v/v of LC-MS grade formic acid was added just before infusion in the mass spectrometer.

The ESI mass spectra were acquired through direct infusion at 7 µL min^{-1} flow rate in a TripleTOF® 5600^{+} high-resolution mass spectrometer (Sciex, Framingham, MA, U.S.A.), equipped with a DuoSpray® interface operating with an ESI probe.
The ESI source parameters were as follows: positive polarity, Ionspray Voltage Floating 5500 V, Temperature 0, Ion source Gas 1 (GS1) 35 L/min; Ion source Gas 2 (GS2) 0; Curtain Gas (CUR) 25 L/min, Declustering Potential (DP) 50 V, Collision Energy (CE) 10 V, acquisition range 880-2400 *m/z*.
For acquisition, Analyst TF software 1.7.1 (Sciex) was used and deconvoluted spectra were obtained by using the Bio Tool Kit micro-application v.2.2 embedded in PeakView™ software v.2.2 (Sciex).

ESI mass spectra

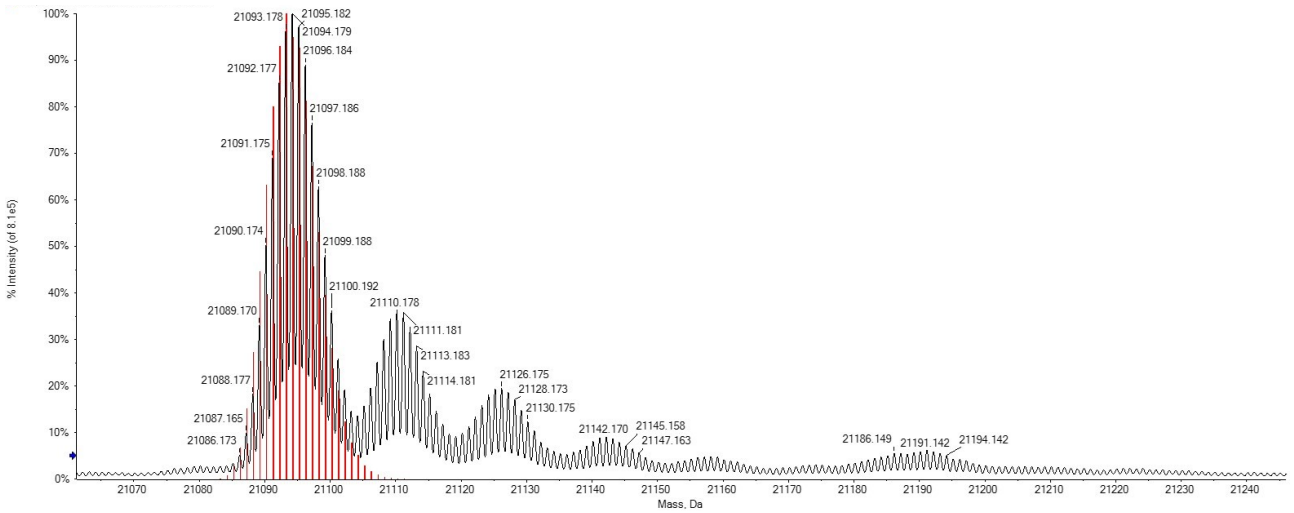

Figure S4. Deconvoluted ESI-Q-TOF mass spectrum of HuHf 10^{-6} M in 2 mM ammonium acetate solution at pH 6.8. Experimental isotopic distribution (black line) vs the theoretical one (red lines) obtained from the HuHf amino acid sequence ($C_{923}H_{1425}N_{259}O_{295}S_7$) Measured: 21094.179 Da; theoretical: 21094.274 Da (calculated on the most abundant theoretical isotope peak).

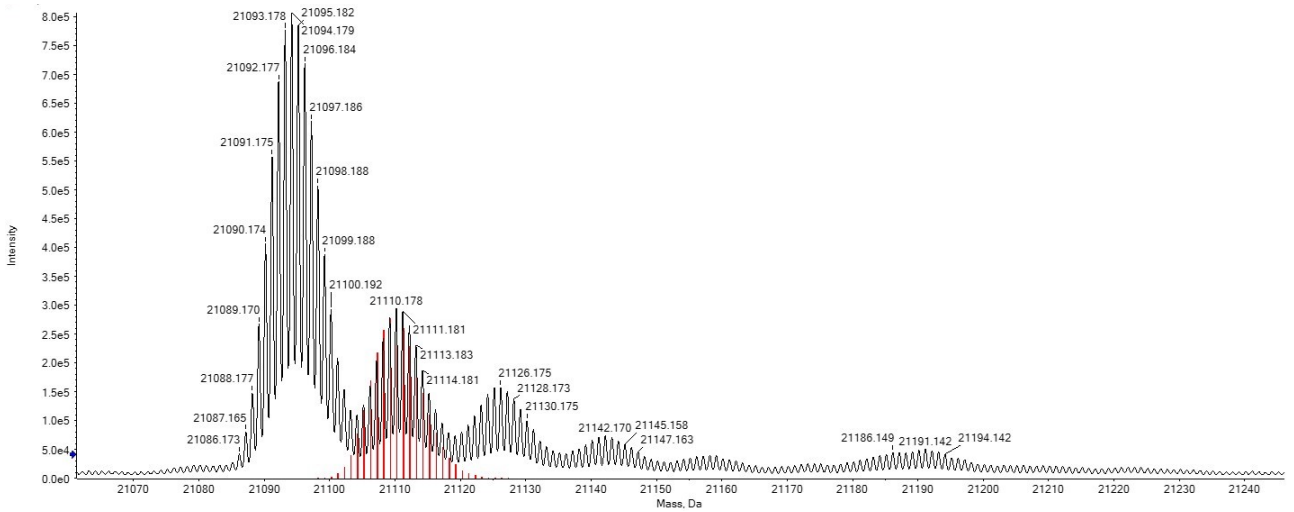

Figure S5. Deconvoluted ESI-Q-TOF mass spectrum of HuHf 10^{-6} M in 2 mM ammonium acetate solution at pH 6.8. Experimental isotopic distribution (black line) vs the theoretical one (red lines) obtained from the HuHf amino acid sequence with one oxidized Met residue ($C_{923}H_{1425}N_{259}O_{296}S_7$) Measured: 21110.178 Da; theoretical: 21110.279 Da (calculated on the most abundant theoretical isotope peak).

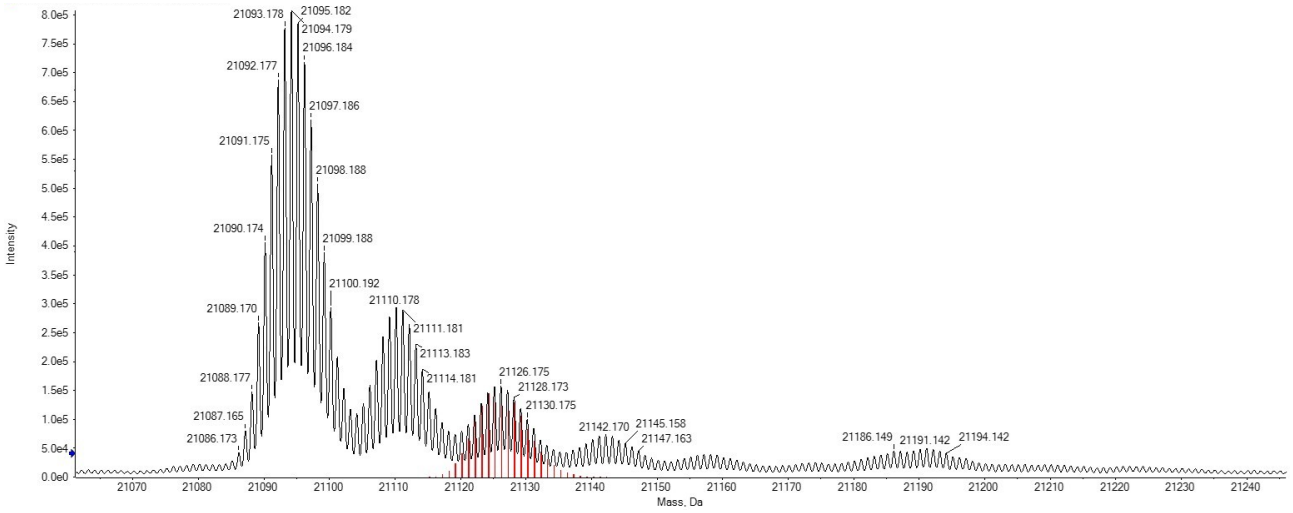

Figure S6. Deconvoluted ESI-Q-TOF mass spectrum of HuHf 10^{-6} M in 2 mM ammonium acetate solution at pH 6.8. Experimental isotopic distribution (black line) vs the theoretical one (red lines) obtained from the HuHf amino acid sequence with two oxidized Met residues ($C_{923}H_{1425}N_{259}O_{297}S_7$) Measured: 21126.175 Da; theoretical: 21126.274 Da (calculated on the most abundant theoretical isotope peak).

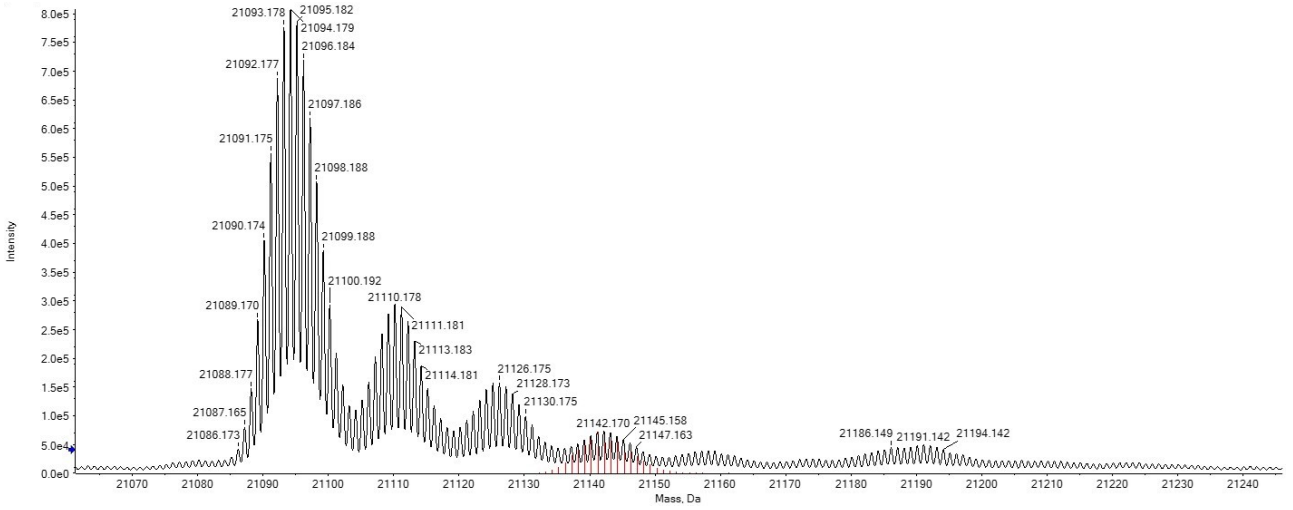

Figure S7. Deconvoluted ESI-Q-TOF mass spectrum of HuHf 10^{-6} M in 2 mM ammonium acetate solution at pH 6.8. Experimental isotopic distribution (black line) vs the theoretical one (red lines) obtained from the HuHf amino acid sequence with three oxidized Met residues ($C_{923}H_{1425}N_{259}O_{298}S_7$) Measured: 21142.170 Da; theoretical: 21142.269 Da (calculated on the most abundant theoretical isotope peak).

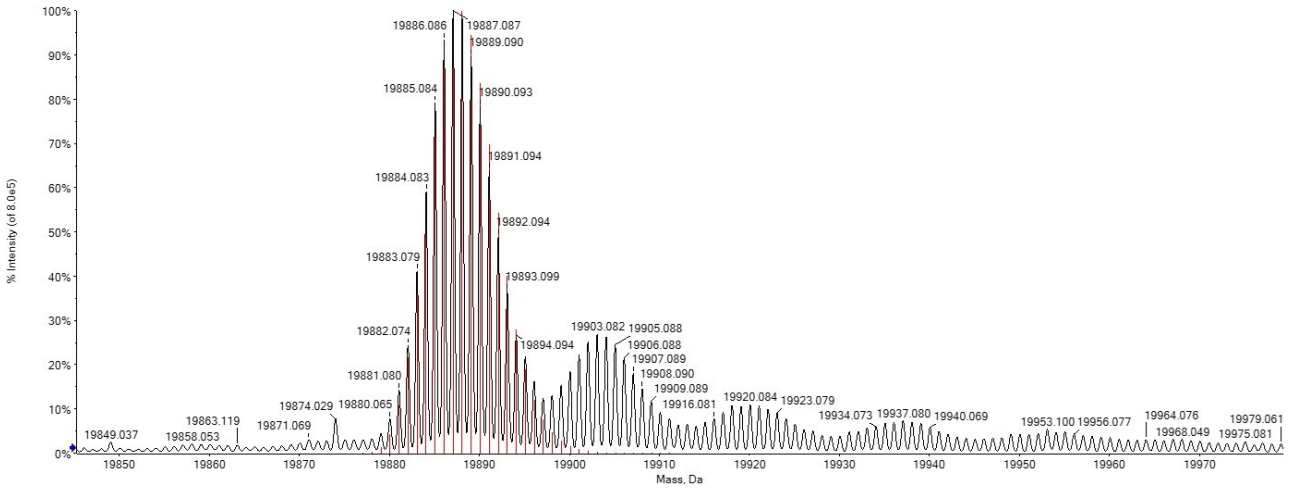

Figure S8. Deconvoluted ESI-Q-TOF mass spectrum of HuLf 10^{-6} M in 2 mM ammonium acetate solution at pH 6.8. Experimental isotopic distribution (black line) vs the theoretical one (red lines) obtained from the HuLf amino acid sequence ($C_{885}H_{1382}N_{244}O_{268}S_5$) Measured: 19887.087 Da; theoretical: 19887.091 Da (calculated on the most abundant theoretical isotope peak).

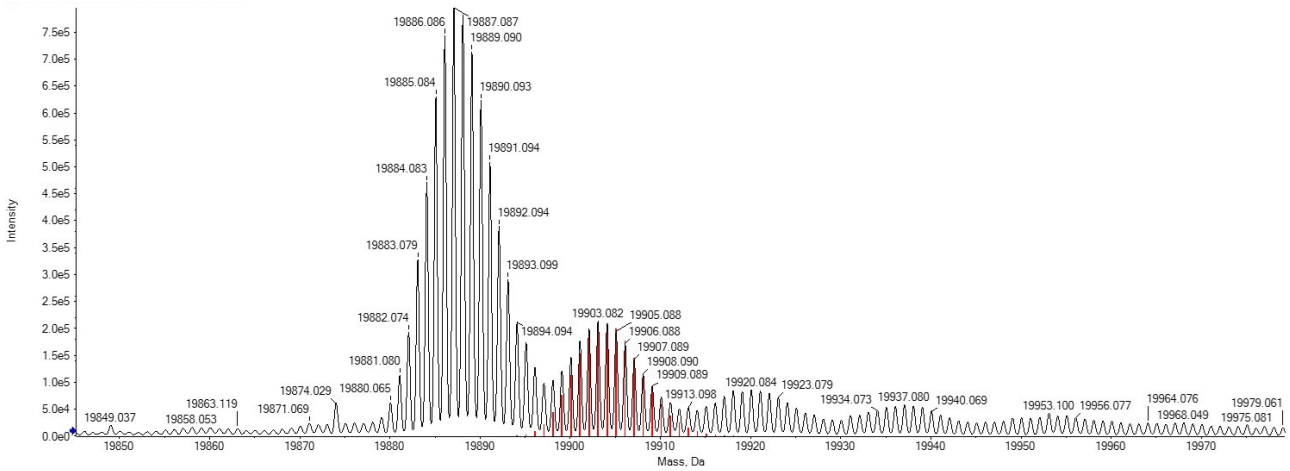

Figure S9. Deconvoluted ESI-Q-TOF mass spectrum of HuLf 10^{-6} M in 2 mM ammonium acetate solution at pH 6.8. Experimental isotopic distribution (black line) vs the theoretical one (red lines) obtained from the HuLf amino acid sequence with one oxidized Met residue ($C_{885}H_{1382}N_{244}O_{269}S_5$) Measured: 19903.082 Da; theoretical: 19903.086 Da (calculated on the most abundant theoretical isotope peak).

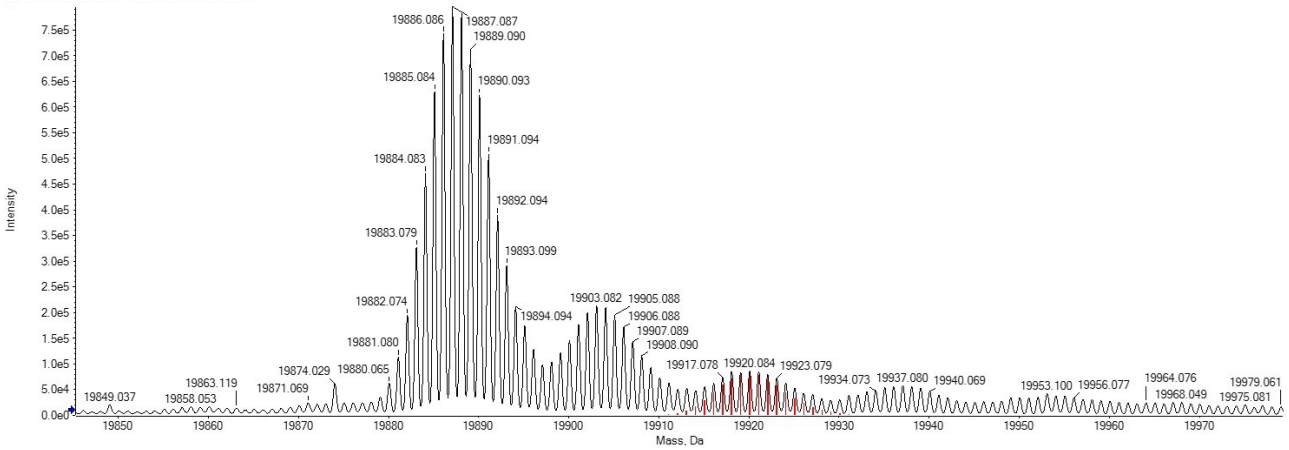

Figure S10. Deconvoluted ESI-Q-TOF mass spectrum of HuLf 10^{-6} M in 2 mM ammonium acetate solution at pH 6.8. Experimental isotopic distribution (black line) vs the theoretical one (red lines) obtained from the HuLf amino acid sequence with two oxidized Met residues ($C_{885}H_{1382}N_{244}O_{270}S_5$) Measured: 19919.084 Da; theoretical: 19919.081 Da (calculated on the most abundant theoretical isotope peak).

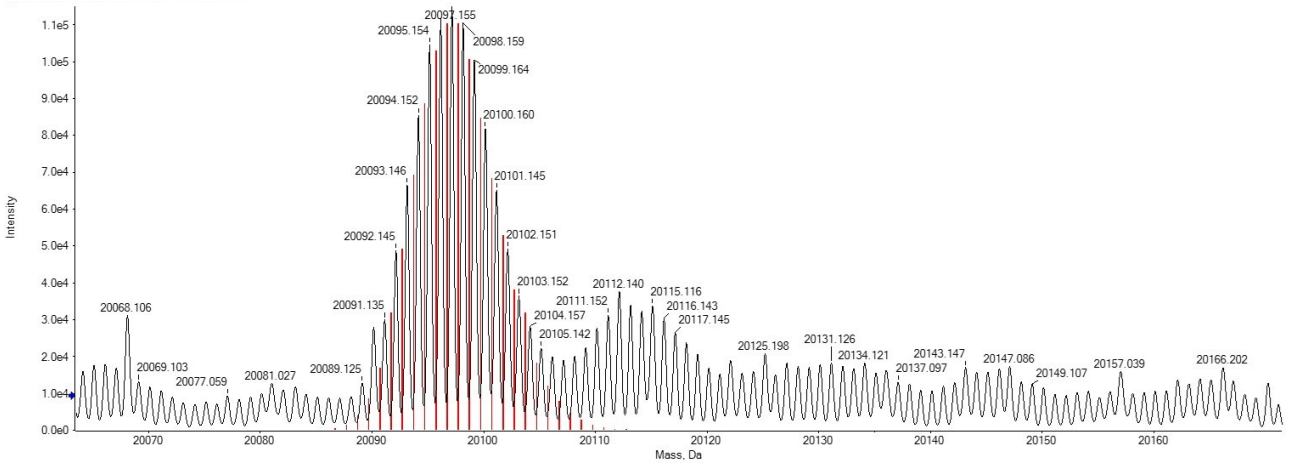

Figure S11. Deconvoluted ESI-Q-TOF mass spectrum of HuLf 10^{-6} M in 2 mM ammonium acetate solution at pH 6.8. Experimental isotopic distribution (black line) vs the theoretical one (red lines) obtained from the adduct [HuLf + $4Fe^{3+}$ - $12H^{+}$] ($C_{885}H_{1368}N_{244}O_{268}S_5Fe_4$) Measured: 20097.155 Da; theoretical: 20097.725 Da (calculated on the most abundant theoretical isotope peak).

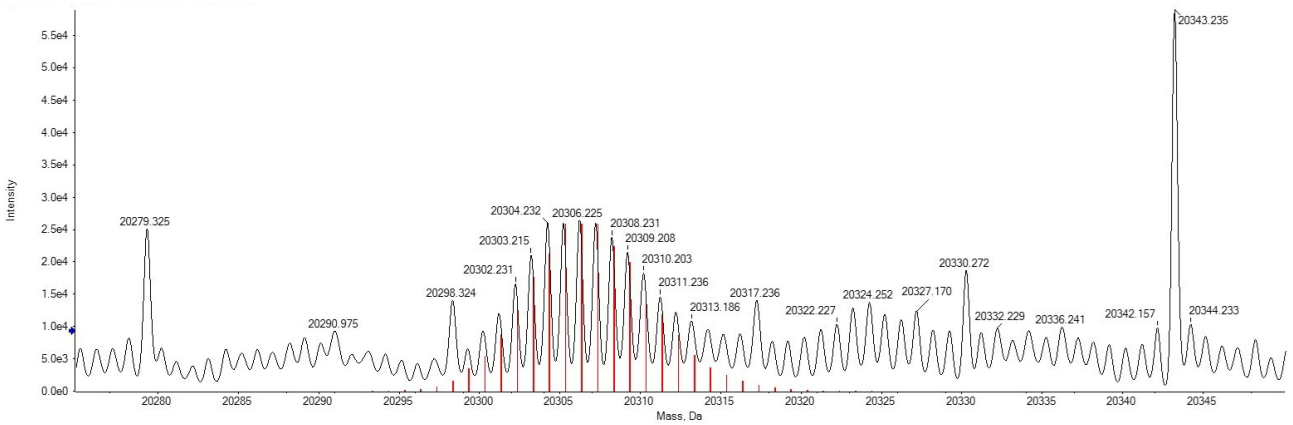

Figure S12. Deconvoluted ESI-Q-TOF mass spectrum of HuLf 10^{-6} M in 2 mM ammonium acetate solution at pH 6.8. Experimental isotopic distribution (black line) vs the theoretical one (red lines) obtained from the adduct [HuLf + $8Fe^{3+}$ - $24H^{+}$] ($C_{885}H_{1354}N_{244}O_{268}S_5Fe_8$) Measured: 20306.255 Da; theoretical: 20306.355 Da (calculated on the most abundant theoretical isotope peak).

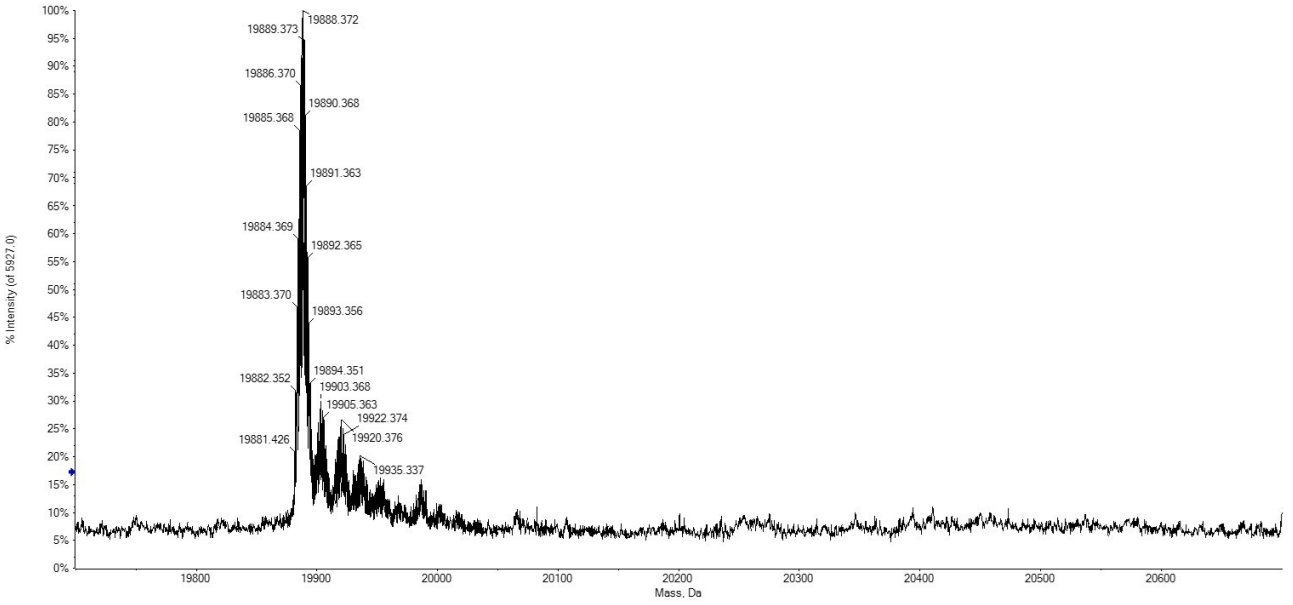

Figure S13. Deconvoluted ESI-Q-TOF mass spectrum of mineralized HuLf 10^{-6} M in 2 mM ammonium acetate solution at pH 6.8, and treated for 15 min with 10 eq. of EDTA.

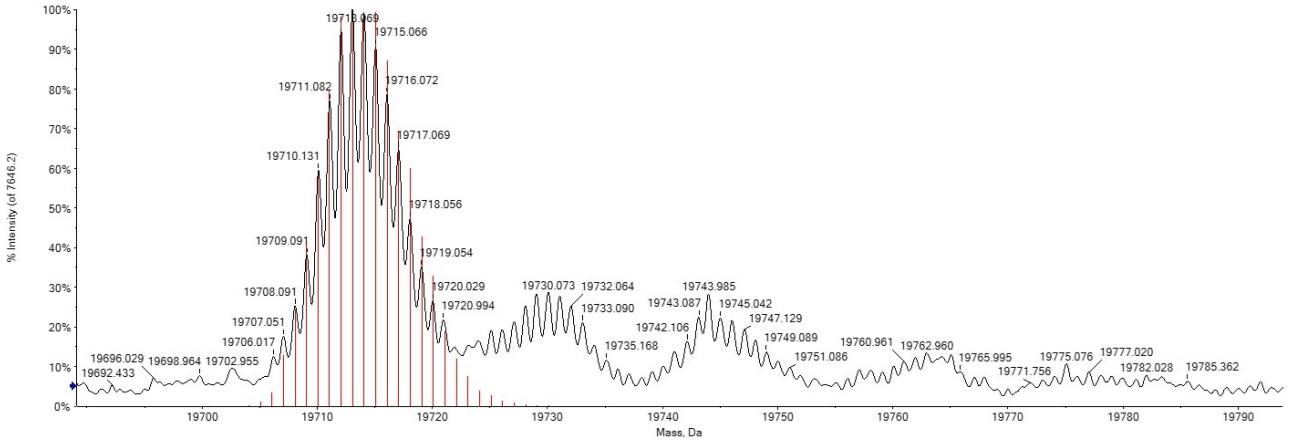

Figure S14. Deconvoluted ESI-Q-TOF mass spectrum of HuLf- E60AE61AE64A mutant 10^{-6} M in 2 mM ammonium acetate solution at pH 6.8. Experimental isotopic distribution (black line) vs the theoretical one (red lines) obtained from the HuLf triple mutant amino acid sequence ($C_{879}H_{1376}N_{244}O_{262}S_5$) Measured: 19714.069 Da; theoretical: 19714.077 Da (calculated on the most abundant theoretical isotope peak).

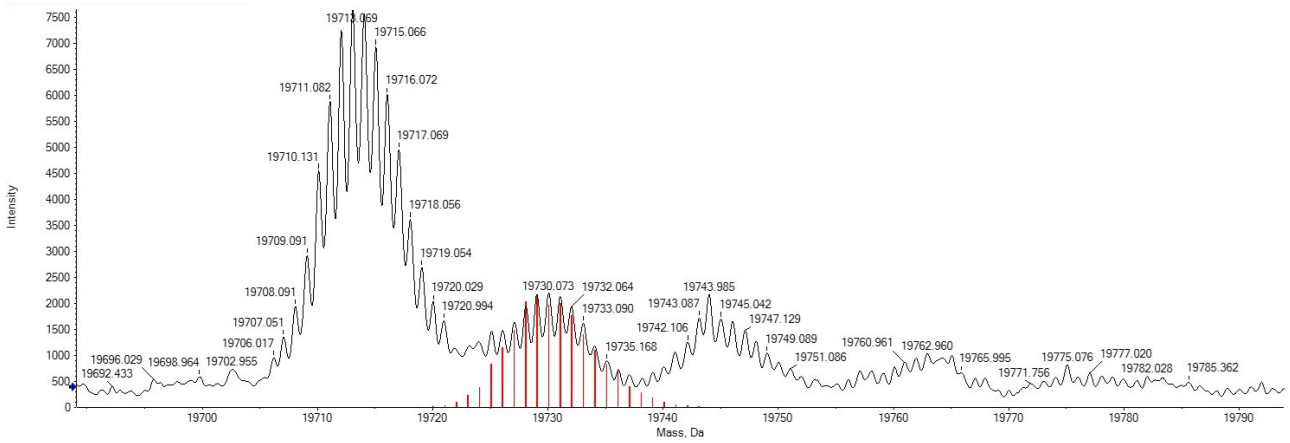

Figure S15. Deconvoluted ESI-Q-TOF mass spectrum of HuLf-E60AE61AE64A mutant 10^{-6} M in 2 mM ammonium acetate solution at pH 6.8. Experimental isotopic distribution (black line) vs the theoretical one (red lines) obtained from the HuLf triple mutant amino acid sequence with one oxidized Met residue ($C_{879}H_{1376}N_{244}O_{263}S_5$) Measured: 19730.073 Da; theoretical: 19730.072 Da (calculated on the most abundant theoretical isotope peak).

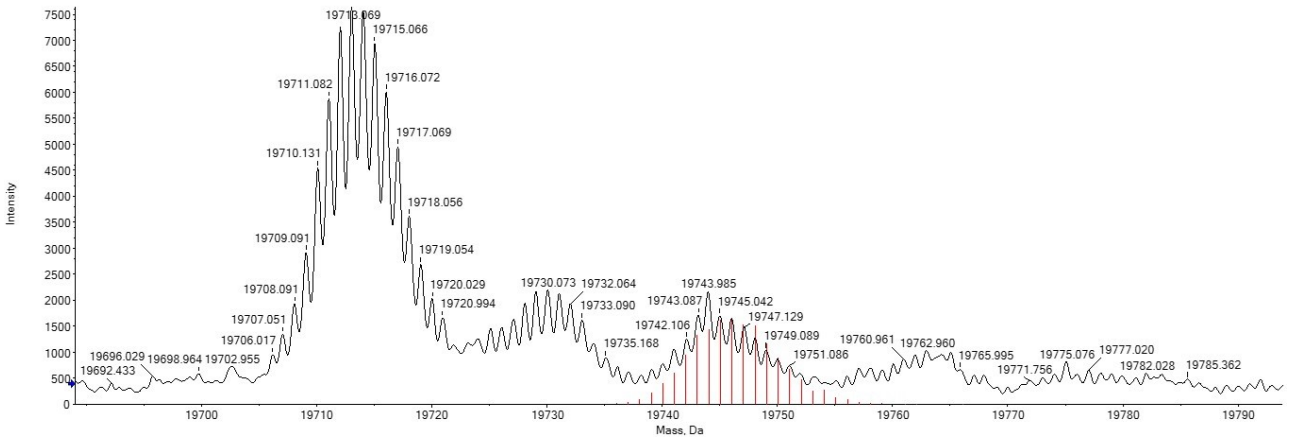

Figure S16. Deconvoluted ESI-Q-TOF mass spectrum of HuLf-E60AE61AE64A mutant 10^{-6} M in 2 mM ammonium acetate solution at pH 6.8. Experimental isotopic distribution (black line) vs the theoretical one (red lines) obtained from the HuLf triple mutant amino acid sequence with two oxidized Met residue ($C_{879}H_{1376}N_{244}O_{264}S_5$) Measured: 19746.045 Da; theoretical: 19746.067 Da (calculated on the most abundant theoretical isotope peak).

References

1 C. Pozzi, S. Ciambellotti, C. Bernacchioni, F. Di Pisa, S. Mangani and P. Turano, *Proc. Natl. Acad. Sci. U. S. A.*, 2017, **114**, 2580–2585.

2 C. Pozzi, F. Di Pisa, C. Bernacchioni, S. Ciambellotti, P. Turano and S. Mangani, *Acta Crystallogr. Sect. D Biol. Crystallogr.*, 2015, **71**, 1909–1920.

3 S. Ciambellotti, C. Pozzi, S. Mangani and P. Turano, *Chem. - A Eur. J.*, 2020, **26**, 5770–5773.

4 R. E, C. S, C. L, M. T, K. T, B. C, G. S, F. M, T. P and L. C, *Angew. Chem. Int. Ed. Engl.*, 2016, **55**, 2446–2449.